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Review

Cationic polymeric nanoformulation: Recent advances in material design for CRISPR/Cas9 gene therapy

Kaifeng Chen[a], Shan Jiang[a], Yun Hong[a], Zibiao Li[b,**], Yun-Long Wu[a,*], Caisheng Wu[a,***]

[a] Fujian Provincial Key Laboratory of Innovative Drug Target Research, State Key Laboratory of Cellular Stress Biology, School of Pharmaceutical Sciences, Xiamen University, Xiamen, China

[b] Institute of Materials Research and Engineering, Agency for Science, Technology and Research, 2 Fusionopolis Way, #08-03 Innovis, Singapore, 138634, Singapore

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ABSTRACT

CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/clustered regularly interspaced short palindromic repeat associated proteins 9) gene editing platform is a promising therapeutic tool for genetic disorders, due to its ability to manipulate the pathogenic gene in genomic level and to easily target specific gene by manipulating single-guide RNA. However, its successful delivery remains a challenge. Up to now, great efforts have been made to explore an effective strategy for CRISPR/Cas9 delivery. But among those delivery methods, physical methods are mainly operated on cultured cells thus limited to laboratorial use; viral vectors are hindered by fetal immunogenic and carcinogenic effects thus dubious in clinical application. Therefore, cationic polymeric vectors, with the ability to interact with CRISPR/Cas9 system to form a nanoformulation as a non-viral approach, are attracting increasing attentions, due to advantages such as well protection of cargos, less limitation in payload size, low immunogenicity or carcinogenicity, potential modifications for further functions, and ease in mass production. In this review, the recent discoveries on polymeric vectors utilized in delivery of CRISPR/Cas9 system will be summarized. With emphasis on advanced features of those polymeric vectors or their nanoformulations to meet the demands of different CRISPR/Cas9 delivery forms (plasmid, mRNA or protein), the detailed illustrations on their disease treatment applications, such as cancer, diabetes or antibiotic-resistant infections, will also be reviewed.

1. Introduction

Gene therapy could manipulate DNA or RNA to treat and prevent human diseases [1]. In the past years, researchers mainly focused on restoring the function of missing gene by exogenous transgene expression or downregulation of pathogenic gene by RNAi molecules. However, the temporary effect of transgene or RNAi hinders its application while the retention of disease-causing gene leads to a prominent negative effect to treatment. Recent developments of a novel gene editing platform-CRISPR/Cas9 technology provide approaches to correct genic disorders in genome level [2]. The system is composed of a nuclease Cas9 and a single guide RNA (sgRNA). Guided by sgRNA, Cas9 can target to a specific site of genome and cause double-strand breaks, which will result in gene replacement or insert/delete mutations respectively [3]. A number of reports have shown its application in gene therapy to correct or disrupt the disease-causing genes and this technology shows great potential in reforming therapy of numerous human diseases including cancer, cardiovascular diseases, virus infection, antibiotic-resistant bacteria infection etc [4–6]. (see Table 1)

However, despite the merits of CRISPR/Cas9 system in gene therapy, the success of its function relies on an effective intracellular co-delivery of its two components: Cas9 nuclease and sgRNA. Cas9 can be delivered in three forms: plasmid, mRNA or protein [7]. Cas9 plasmid system is an appealing delivery strategy attributing to its low-cost manipulation, simplicity and stability. Cas9 together with sgRNA, and even HDR templates are able to be conveniently packed in the same plasmid. However, its application is also hindered by several drawbacks such as the long lag time for nuclear entry or expression as well as high off-target effects on account of persistance expression of plasmid. The delivery of Cas9 mRNA is an alternative option. It leads to quicker gene editing efficiency and lower risk of off-target effect. However, the poor stability of mRNA shadows its application and the short duration time

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: lizb@imre.a-star.edu.sg (Z. Li), wuyl@xmu.edu.cn (Y.-L. Wu), wucsh@xmu.edu.cn (C. Wu).

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Abbreviations

CRISPR	Clustered regularly interspaced short palindromic repeat
Cas9	clustered regularly interspaced short palindromic repeat associated proteins 9
sgRNA	single guide ribonucleic acid
DNA	deoxyribonucleic acid
DSBs	double-strand breaks
HDR	homologous directed repair
NHEJ	non-homologous end-joining
PEI	poly(ethylene imine)
PAMAM	poly(amido amine)
LMWPEI	low molecular weight PEI
HMWPEI	high molecular weight PEI
PLGA	poly(lactic-*co*-glycolic acid)
GO	graphene oxide
CLAN	cationic lipid assisted nanoparticle
PEG	poly(ethylene glycol)
HPV	human papillomavirus
Rb	retinoblastoma protein
RNAi	RNA interference
CML	chronic myeloid leukemia
VEGFA	vascular endothelial growth factor A
MTH1	mutT homolog1
BAFFR	B-cell activating factor receptor
MRSA	methicillin-resistant *Staphylococcus Aureus*

Table 1
A summary of current researches on polymeric nanoformulations for delivery of CRISPR/Cas9 system in gene therapy.

Formulation	Delivery vector	Targeting gene	Cell type	Application	Ref
Plasmid	Branched PEI 25 kDa	Slc26a4 locus	Neuro2a cells		[33]
	JetPEI polymer-based DNA transfection reagent	E7 oncogene	HPV16 positive cervical cancer cells (ect. SiHa, Cask)	Cervical cancer	[34]
	PEI-β-CD	EGFP gene	HeLa-EGFP cells		[35]
	PEG-PEI-Cholesterol lipopolymer	VEGFA gene	OS cell (ect.K7M2)	Osteosarcoma	[36]
	"core-shell" artificial virus	MTH1 gene	SKOV3 cells	Ovarian cancer	[37]
	PPO-NMe3 and Pluronic F127	E7 oncogene	HeLa cells	Cervical cancer	[38]
	Polymeric and hybrid SiO2-coated capsules	dTomato gene	HEK293T-dTomato cells		[39]
	PEGylated nanoparticles based on the cationic α-helical polypeptide	Plk1 gene	HeLa and K562 cells	Cancer	[40]
	Cationic lipid-assisted nanoparticle	BCR-ABL fusion gene	CML cell line K562	Chronic myeloid leukemia	[41]
		B220, BAFFR gene	B cells	rheumatoid arthritis	[42]
		Ntn1 gene	macrophages	T2D	[43]
	PEGylated chitosan copolymers		HEK293 cells		[44]
mRNA	Cationic lipid-assisted nanoparticle	NLRP3 gene	macrophages	Inflammatory diseases	[45]
Protein	Cr-Nanocomplex	mecA gene	MRSA	Antimicrobial	[46]
	GO-PEG-PEI	EGFP gene	AGS-EGFP cell		[47]
	P(Asp-AED-ICA) – PEG		HEK 293 cells MZ-CRC-1 cells RAW 264.7 cells U87-MG cells		[48]
	[p(BAC-TET)]-based polymers		HEK 293 cells HCT 116 cells RAW 264.7 cells		[49]

may limit its gene editing efficiency. The delivery of Cas9 protein together with sgRNA is the most straightforward way to induce CRISPR/Cas9 gene editing. This approach leads to the fastest genome editing as well as the most transient functionality with minimal off-target effects and toxicity. However, the large size of Cas9 protein brings inconvenience to its delivery, as many delivery carriers such as viral vectors are size limited.

In form of plasmid, Cas9 and sgRNA can be encoded in the same plasmid to achieve co-delivery; in form of protein, the positive charged Cas9 can interact with sgRNA and form ribonucleoprotein; while in form of mRNA, the co-delivery should be achieved by vectors. It is worth mentioned that Cas9 exhibits large molecular size in all forms, while the effective condensation or package of Cas9 remains a challenge for vectors. Furthermore, as the function of CRISPR/Cas9 takes place in nucleus, the nuclear transport is necessary for its delivery. Hence, the vectors also have to show protection of the components, especially for sensitive sgRNA. Fortunately, in recent years, a number of delivery strategies were reported, varying from physical to chemical or from viral to non-viral [8–17]. Among those strategies, physical methods depend on naked cargos without any vectors' protection. When administered directly *in vivo*, the naked cargos are vulnerable to enzymatic degradation and rapid clearance, which are limited to laboratorial use. For viral vectors, though those adeno-associated virus and lentivirus exhibit excellent gene transfection efficiency, their applications are hindered by their limited packaging size, potential but fetal immunogenic as well as carcinogenic effects [18–20].

Therefore cationic polymeric vectors, that exhibit low immunogenicity or carcinogenicity, protection of cargos, less limitation in payload size, ease of production in large-scale, and ability to form nanoformulation with CRISPR/Cas9 system, are excellent alternatives [21]. In addition, polymers can achieve enhanced circulation time, cell or tissue specific delivery and controlled release through further modification [22–32]. The above properties make polymeric vectors a promising option for delivery of CRISPR/Cas9 platform in gene therapy. In this review, we intend to summarize the current polymeric vectors that are applied in CRISPR/Cas9 delivery and their applications in treatment for human diseases as shown in Fig. 1, which are important but not fully summarized yet.

2. Cationic polymers applied in CRISPR/Cas9 delivery

2.1. Cationic polymers applied in Cas9 plasmid delivery

Cas9 plasmid system is an appealing delivery strategy attributing to its low-cost manipulation and simplicity. Cas9 together with sgRNA, and even HDR templates are able to be conveniently packed in the same plasmid. Moreover, plasmids exhibit higher stability compared with proteins or mRNAs. However, plasmid system also suffers from several drawbacks. Firstly, the nuclear entry and expression of plasmids require for a long lag time before the therapeutic efficacy. Secondly, a long persistence of the plasmids leads to the persistence of Cas9, which leads to higher off-target effects. Thirdly, the requirement of nuclear entry of

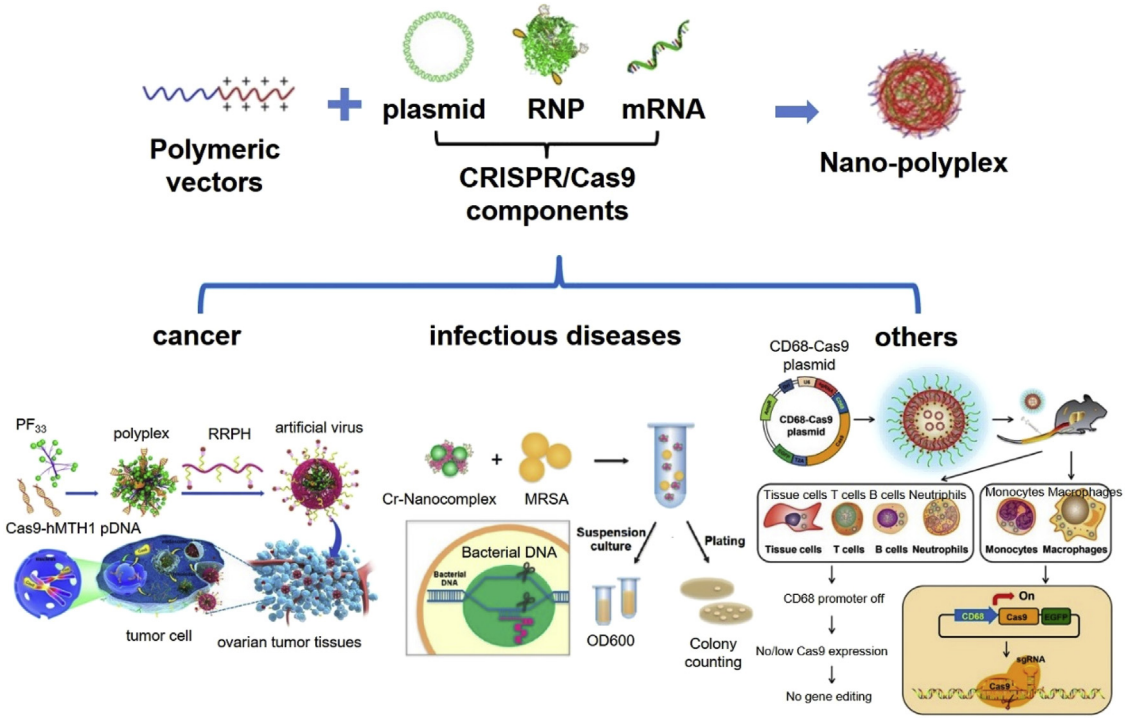


Fig. 1. Schematic diagram of structure of cationic polymeric nanoformulation and their application in CRISPR/Cas9 system delivery for gene therapy and disease treatments.

plasmids may lead to decreased genome editing efficiency.

Numerous cationic polymers were studied to delivery gene in the past decade, among which some pioneers have been acknowledged as classical transfection agents and widely used in the world, such as poly (ethylene imine) (PEI). Those polymers possess high positive charge to condense DNA and protect DNA from DNase and other physical and chemical factors. The positive charge also endows the polymers with high endosome escape to release the plasmid. Up to now, several polymers have been tested to delivery CRISPR/Cas9 plasmid such as poly(ethylenimine) (PEI), poly(amido amine) (PAMAM), polypeptide, chitosan et al. and they exhibit good genome editing potential.

2.1.1. PEI based polymers in CRIEPR/Cas9 plasmid delivery

PEI, considered as a leading material for polymeric gene delivery since the first successful application by Boussif et al. in 1995 [50], is widely used as an effective transfection reagent and commercially available at present, such as branched PEI and linear PEI, as shown in Fig. 2A. In addition, many PEI based polymeric vectors have been applied in gene therapy clinical trials so far for their high transfection efficiency and potential for further modification like BC819/PEI [51], phIL-12/polyethyleneglycol–polyethyleneimine–cholesterol (PPC) [52] and so on. The high concentration of amino groups endows PEI with a high positive charge to strongly interact with plasmid and a strong proton sponge effect to effectively escape from endosomes, thus it possesses high gene transfection efficiency. It has been demonstrated that simple 25-kDa branched PEI and commercial jet-PEI polymer-based DNA transfection reagent could achieve delivery of Cas9/sgRNA encoded plasmid successfully, according to studies respectively carried out by Kim et al. [33] and Wang et al. [34]. However, its application is shadowed by its high cytotoxicity as the research showed the decreased cell viability down to 67.57%, at optimal N/P (nitrogen in polymer to phosphor in plasmid) ratios of branched PEI, which called for further improvement of the cationic polymeric structure [33].

It was reported that both the toxicity and transfection efficiency of PEI were positively relative to the molecular weight. Lower transfection efficiency is tolerable considering that a high level expression of Cas9 leads to high level off-target effect, so low molecular weight PEI (LMWPEI) is worth trying in CRISPR/Cas9 plasmid delivery. In a research carried out by Ping et al., LMWPEI was assembled with β-cyclodextrin through host–guest interaction. The complex showed similar structure to high molecular weight PEI (HMWPEI), thereby exhibiting effective transfection efficiency. More importantly, as compared with HMWPEI, the much lower cytotoxicity nature allowed for high-dose-level, repeated transfection. The β-cyclodextrin also enabled the polymer for further modification to endow functions such as blood stability and targeting capability to improve its delivery efficiency, as showed in Fig. 2B [35].

Furthermore, further modifications on PEI can endow the polymers with different functions such as biocompatibility, targeting ability for superior gene delivery. A group of researchers functionalized LMWPEI with polyethyleneglycol (PEG) and cholesterol (CHOL), obtaining a kind of lipopolymer [36]. CHOL served as a biocompatible hydrophobic lipid anchor to enhance the permeability through membrance [53] and PEG served as a biocompatible hydrophilic segment to enhance the stability and hemocompatibility of the lipopolymer. In addition, researchers also modified the lipopolymer with cell-specific aptamers to achieve target delivery of CRISPR/Cas9 system. Fluorination was also an alternative way to modify LMEPEI [54]. The polymer became both hydrophilic and hydrophobic after fluorinated, endowing it with high phase-separation ability in both polar and nonpolar situation. So the polymer had high affinity to lipid bilayer and could be easily taken up by cells as well as escaped form endosomes. Li et al. coated fluorinated LMWPEI with multifunctional RGD-R8-PEG-HA copolymer and formed a polymeric “core-shell” complex for CRISPR/Cas9 target delivery [37]. The peptide RGD-R8 had high affinity to a kind of integrin receptor overexpressed in tumor and tumor vessel, endowing the polymer with tumor targeting ability. The PEG segment could make the complex more stable and the anionic HA segment could reverse the cationic charge of fluorinated LMWPEI to reduce nonspecific interaction with compounds in human body. Furthermore, after HA degradation by abundant hyaluronidase in cancer tissue, the cationic components inside would expose, leading to enhanced cellular uptake and endosome

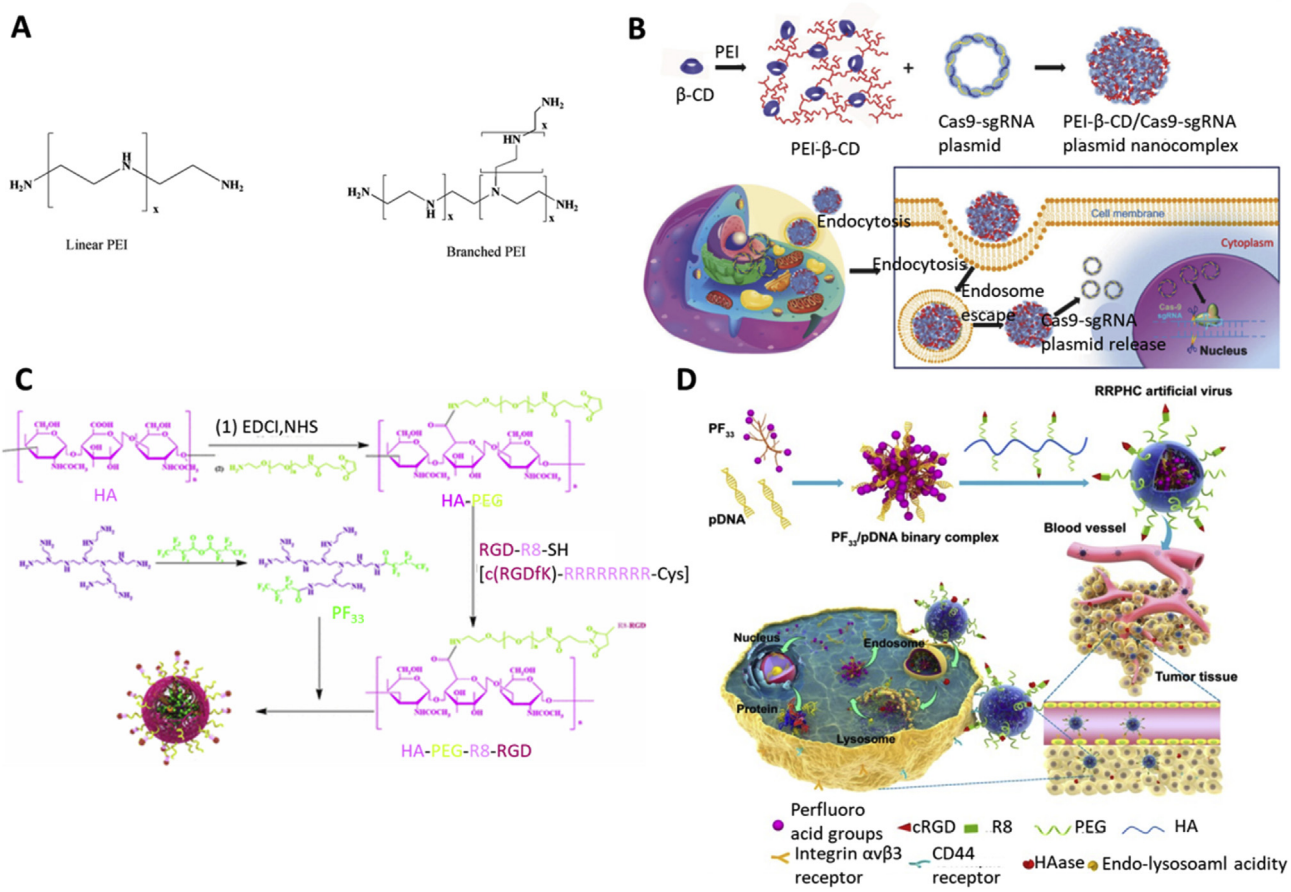


Fig. 2. (A) The chemical structure of linear PEI and branched PEI. (Copyright 2015, Mary Ann Liebert, Inc) [55] (B) The schematic diagram of PEI-β-CD induced CRISPR/Cas9 delivery. The polymer condenses plasmid through electrostatic interaction, forming polymer/plasmid complex. The complex could be taken up through endocytosis and escape from endosome by proton sponge effect [35]. (Copyright 2018, WILEY-VCH) (C) The synthesis process of fluorinated LMWPEI and RGD-R8-PEG-HA copolymer. (Copyright 2016, American Chemical Society) [37] (D) The schematic diagram of the core-shell polymer complex induced CRISPR/Cas9 delivery. Fluorinated LMWPEI condensed plasmid to form the positive charged core and it was coated with multifunctional copolymer shell. The complex target to tumor cells and the core could be exposed in tumor tissue to enhance cellular uptake and endosome escape (Copyright 2017, Ivyspring International Publisher). [56].

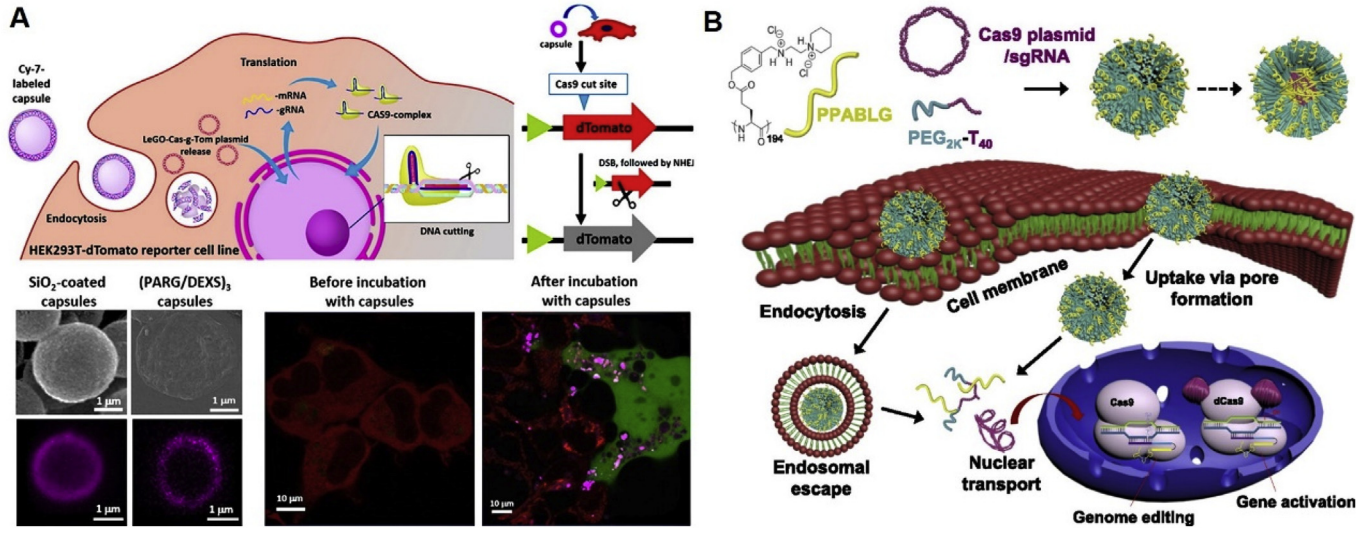


Fig. 3. (A) Illustration of polypeptide/dextran capsule induced CRISPER/Cas9 intracellular delivery and genome editing. The system caused DSBs which were further repaired by NHEJ in dTomato gene, and often resulted in gene silencing. Left bottom: The TEM images of SiO_2 coated and uncoated capsules showed that the inorganic shell strongly enhanced the mechanical strength of the capsules. Right bottom: The CLSM images of gene editing efficiency in HEK293T-dTomato reporter cells. The dTomato protein showed red signal, eGFP plasmid loaded in the capsule with CRISPER/Cas9 plasmid showed the green signal, Cy-7 in the capsules showed the violet signal [39]. (Copyright 2017, Elsevier Inc) (B) Schematic diagram of the formation of polypeptide based nanoparticles and intracellular delivery of Cas9 expression plasmid and sgRNA. The polypeptide condensed CRISPR/Cas9 plasmid and formed nanoformulation together with PEG. The vector could be taken up by cells through endocytosis as well as pore formation, thus leading to effective gene editing. (Copyright 2018, PNAS) [40].

escape, as showed in Fig. 2C–D.

Besides to LMWPEI, other low-charge-density polymers were also reported. Recently, Lao et al. modified poly(propylene oxide) (PPO) for CRISPR/Cas9 plasmid delivery [38]. The quaternary ammonium were added to the terminal of PPO to endow it with DNA loading ability and the polymer was mixed with an amphiphilic tri-block copolymer Pluronic F127 to form self-assembled micelles. The system targeted E7 gene in human papillomavirus and showed efficient gene knockdown, which represented a positive example of cationic polymeric vectors.

2.1.2. Polypeptide based polymers in CRISPR/Cas9 plasmid system delivery

Polypeptides, in which amino acids are linked through peptides bonds, are protein -mimicking polymers that are biodegradable and biocompatible. When composed of alkaline/acidic amino acids or modified with charged groups, those polymers are able to condense oppositely charged drugs and biomolecules, such as genes [57]. It has already confirmed that polyarginine [58], polylysine and other polycationic amino acids were qualified for gene delivery and recently they were tested for CRISPR/Cas9 plasmid system. Timin et al. designed a polymeric capsule consisting of polyarginine and dextran [39]. The capsule was formed through layer-by-layer electrostatic self-assembly and exhibit excellent stability, large loading ability and good biocompatibility compared with other vectors, as showed in Fig. 3A. Some peptides with specific functions could be decorated for gene delivery, remaining their bio-functions for enhanced delivery efficiency. Wang et al. modified a kind of α-helical polypeptide which possessed helicity-associated cell-penetrating ability with some cationic side-chain groups for plasmid/sgRNA delivery [40]. The polypeptide could be taken up by cells through endocytosis as well as pore formation, as showed in Fig. 3B. The researchers demonstrated that the cell-penetrating ability of this polypeptide could induce stronger cellular uptake.

2.1.3. Cationic lipid assisted PLGA nanoparticles in CRISPR/Cas9 plasmid delivery

Poly(lactic-*co*-glycolic acid) (PLGA) is a kind of widely used biodegradable polymers as its hydrolysates are glycolic acid and lactic acid that can be easily metabolized by human bodies, as showed in Fig. 4A [59]. Few toxicity effects are found in the use of PLGA in human body, so the application of PLGA as delivery systems and biomaterials has been approved by the US FDA. What's more, the degradation time of PLGA can be changed from days to years through tuning the relative ratio of glycolic and lactic acid or the molecular weight of the copolymer therefore achieving a sustained release of its contents [60–64].

Those above properties make PLGA an efficient sustained release delivery system for drugs and genes. However, one main drawback of PLGA is its negative charge that leads to poor cellular uptake as well as endosome escape [65]. In order to enhance the permeability of PLGA particles, positive charged agents are added into the system, such as cationic lipids [66]. Lipids have been utilized as delivery systems in the shape of liposomes for their easy preparation, high biocompatibility and variable surface modification and it is reported that lipid can be applied combined with polymers, resulting in lipopolyplexes and lipid-polymer hybrid nanoparticles which show both advantages. Recently a serious of researches were carried out by Wang et al. that applied a kind of cationic cholesterol assisted PLGA nanoparticles (CLAN) as CRISPR/Cas9 plasmid delivery systems, as showed in Fig. 4B [41–43]. Those nanoparticles composited of a PEG modified PLGA/cationic cholesterol were prepared and exhibited gene editing efficiency in macrophages, CML cells and B cells.

2.1.4. Chitosan in CRISPR/Cas9 plasmid delivery

Chitosan is a kind of natural polysaccharide composited of repeating units of glucosamine and N-acetyl-glucosamine. It is widely considered as a nontoxic and biodegradable polymer with positive charge thus suitable for gene delivery vectors [67]. In addition, chitosan is mucoadhesive. Mucus is a blend of molecules such as salts, lysozyme, mucins and exhibits high viscoelastic properties and negative charge in physiological condition, affecting drugs and particles permeability by forming physical barrier as well as interacting electrostatically with cationic polymers. With mucoadhesion, chitosan shows greater potential in mucosal delivery. So far it has been demonstrated by Zhang et al. that PEGylated chitosan nanocomplexes loaded with Cas9 plasmids exhibited high mucoadhension and remained most physicochemical properties after nebulization, indicating the feasibility to deliver CRISPR/Cas9 system by inhalation, as showed in Fig. 5 [44].

2.2. Cationic polymers applied in Cas9 mRNA delivery

The delivery of Cas9 mRNA is an alternative option. The use of mCas9 leads to relatively quick gene editing efficiency as it avoids the need for nuclear entry and DNA transcription. This strategy also decreases the risk of potential off-target effects because the quick clearance of mRNA results in transient expression of Cas9. In addition, *in vitro* transcription provides an easy way for manipulation of mRNA. However, the poor stability of mRNA shadows its application and the short duration time may limit its gene editing efficiency [68,69].

In this condition, the properties of polymers like high transfection efficiency and protection form degradation make them suit the demands for Cas9 mRNA delivery. Recently Wang et al. developed a kind of cationic cholesterol assisted PLGA nanoparticles (CLAN) composed of PEG-PLGA and BHEM-chol as CRISPR/Cas9 mRNA delivery systems, as shown in Fig. 6 [45]. The cationic lipids were to neutralize the negative charge of the polymer and the nanoparticles were covered with PEG for prolonged circulation. To fabricate the suitable CLAN, they designed a library of CLANs with different PEG densities and surface charges and then tested the *in vivo* macrophage uptake to screen the optimized. The results showed that the nanoparticle with the highest surface charge and relatively lower PEG density exhibited the best macrophage uptake.

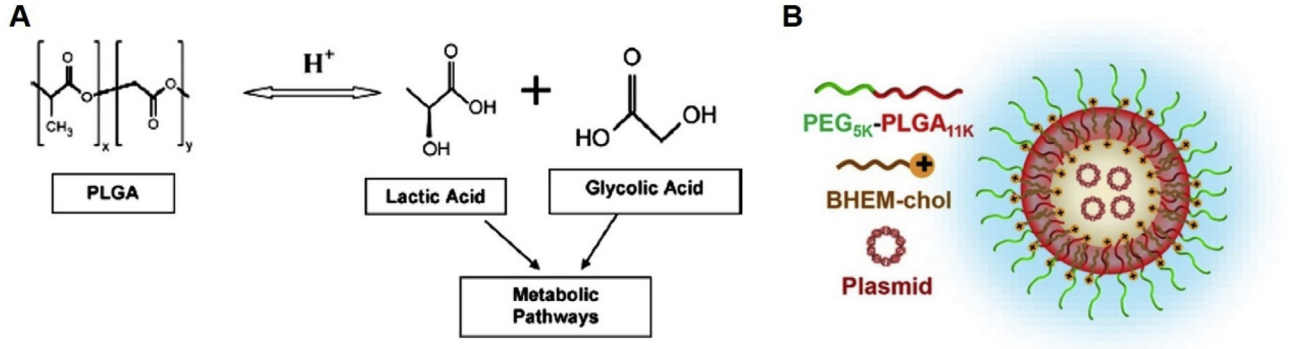
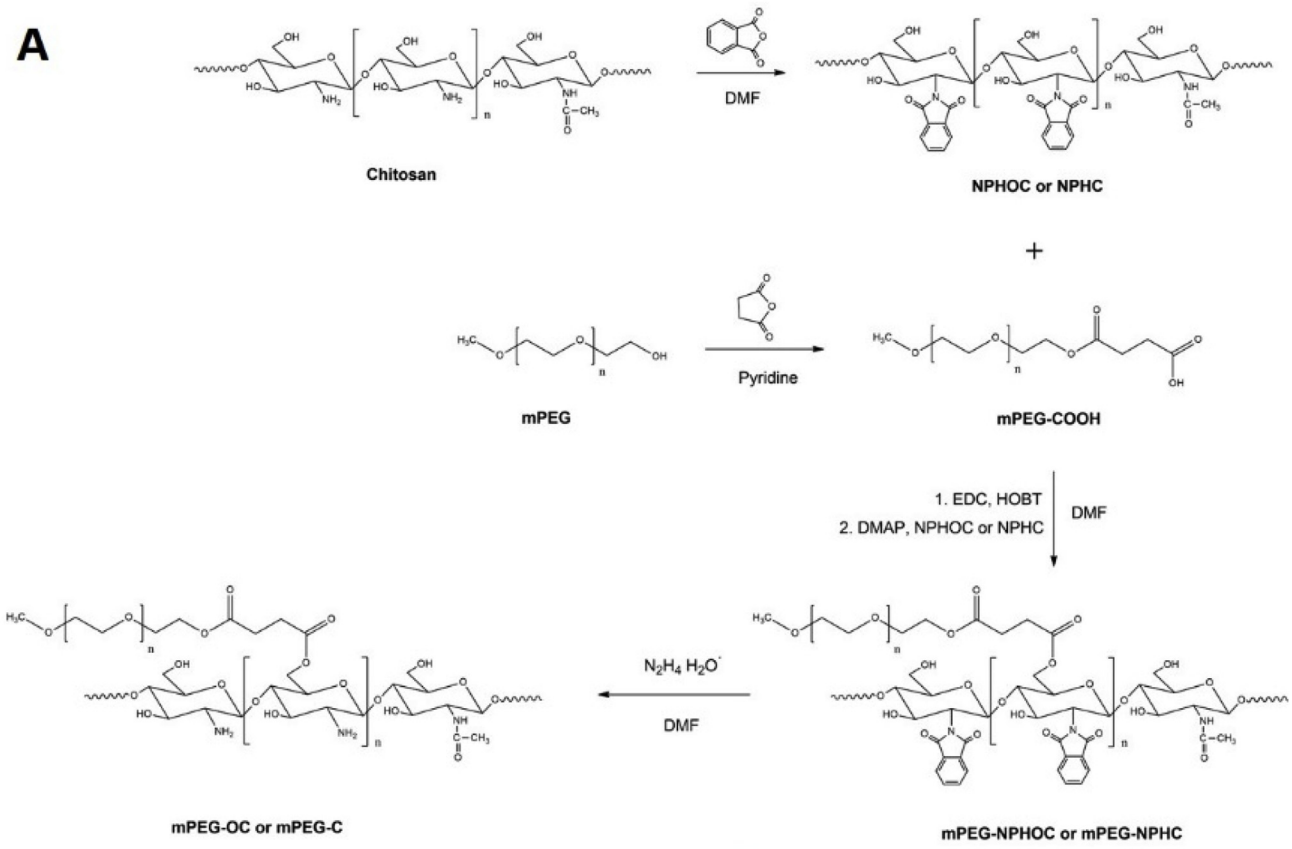


Fig. 4. (A) The metabolism pathway of PLGA. (Copyright 2012, Elsevier B.V.) [59] (B) The diagram of cationic-BHEM assisted PEG-PLGA nanoparticles. (Copyright 2018, American Chemical Society) [43].



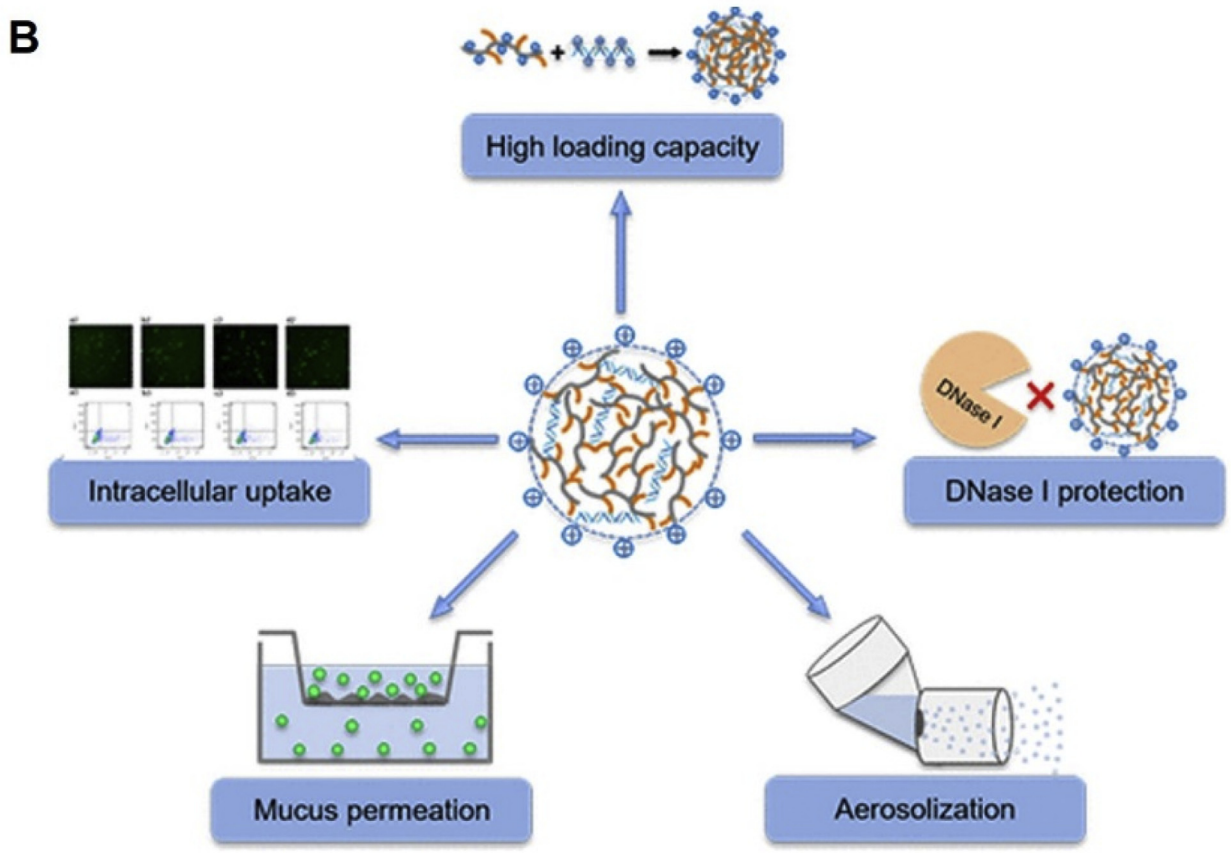


Fig. 5. (A) Synthesis of PEGylated Chitosan. (Copyright 2018, American Chemical Society) [44] (B) The properties of the PEGylated chitosan/plasmid nanocomplex. The nanocomplex exhibits high loading capacity, protection of payloads from DNase I, mucus permeation and aerosolization ability and effective intracellular uptake. (Copyright 2018, American Chemical Society) [44].

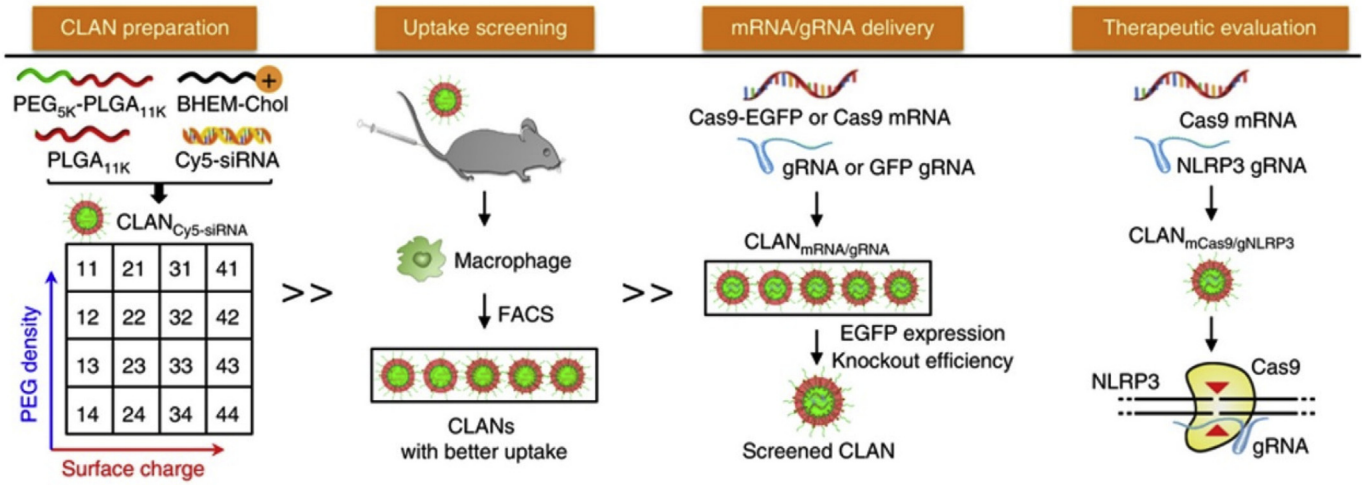


Fig. 6. Illustration diagram of optimizing the suitable CLAN for deliverying mCas9/gRNA to macrophages. A series of CLANs were fabricated by tuning the weight of BHEM-chol to change the surface charge and the fraction of PEG-PLGA to change the PEG density. The celluar uptake of $CLAN_{Cy5\text{-}siRNA}$ was preliminarily tested by fluorescence-actived cell sorting. CLANs with better uptake were further loaded with Cas9-EGFP/Cas9 mRNA and sgRNA and tested for EGFP expression and EGFP knockout efficiency. In the end, the optimized CLAN was used to encapsulate mCas9/sgRNA to target NLRP3 gene in macrophages. (Copyright 2019, Springer Nature Publishing AG) [45].

It was reasonable because high positive charge led to strong interaction with cyto-membrane. However, PEG density was not the lower the better because the shield of positive charge decreased the interaction with cell membrance and enhanced the stability and hemo-compatibility as well.

2.3. Cationic polymers applied in Cas9 protein delivery

The most straightforward way to induce CRISPR/Cas9 gene editing is to directly delivery Cas9 protein together with sgRNA. This approach leads to the fastest genome editing as well as the most transient functionality with minimal off-target effects and toxicity, as it requires no transcription and/or translation. However, pure active Cas9 proteins are more difficult to obtain and more unstable compared with Cas9 plasmid or mRNA. Moreover, the large size of Cas9 protein brings inconvenience to its delivery, as many delivery carriers such as viral vectors are size limited. Therefore polymeric vectors which interact with its payloads though electrostatic interaction or other intermolecular interactions instead of packing the payloads inside are good alternatives for Cas9 protein delivery.

PEI was one of the several polymers first applied in Cas9 protein delivery. In a research carried out by Choung et al. [46], PEI was covalently linked to Cas9 for the use of a minimal amount of polymers. It also avoided the process of encapsulation and a release step of the cargo. Then the polymer-modified Cas9 packaged sgRNA, forming CRISPR complex system and was applied in combating bacterial antibiotic resistance, as showed in Fig. 7A. In another research, Xing et al. modified graphene oxide (GO) with PEI and PEG for Cas9 delivery [47]. In this system, sgRNA would be well protected from enzymatic degradation through electrostatic interactions with modified PEI and π-stacking interactions with rigid GO plane, thus the stability was strongly improved. The vector was demonstrated able to induce gene knock down in mammalian cells, as shown in Fig. 7B.

Apart from PEI based vectors, Gong et al. designed a series of redox-responsive copolymer for delivery of CRISPR/Cas9 ribonucleoprotein [48,49]. Those polymers had some segments in common. The disulfide bonds in the main chain endowed them with redox-responsiveness to release the payloads rapidly in cytosol where the GSH level is relatively high. The imidazole side groups that exhibited high positive charge enabled rapid endosomal escape and PEG segments could shield the surface charge thus enhance circulation time, as shown in Fig. 7C. Moreover, the copolymers can be further modified with side chain such as adamantine and β-cyclodextrin, between which the host-guest interaction strongly enhanced the stability of the complexes and benefited its application, as shown in Fig. 7D.

3. Applications of cationic polymer based CRISPR/Cas9 system in gene therapy

As a recently developed gene editing system, CRISPR/Cas9 system is a promising tool in gene therapy. The system can correct genetic defects by knocking down the target gene or replacing it with donor DNA, thus it can be applied in many genetic disorders and nonmonogenic diseases, such as cancers, cardiovascular diseases, and metabolic disorders. As mentioned before, the effective intracellular delivery of CRISPR/Cas9 system is the premise of its effective therapeutic action. Polymers, that endowed with high transfection efficacy, protection of payloads, low immunogenic and carcinogenic effects, are effective delivery vectors for CRISPR/Cas9 gene therapy applications.

3.1. Cationic polymer based CRISPR/Cas9 system in cancer gene therapy

Cancer is one of the foremost threats to human health nowadays and it's acknowledged that cancer is a kind of genome related disease, which occurs and advances due to a series of mutants in proto-oncogenes and anti-oncogenes. Therefore, it has been proven an effective method for cancer treatment by delivering therapeutic agents to correct the genetic disorders. As a gene editing platform, CRISPR/Cas9 system can silence oncogene expression, correct mutation, suppress tumor angiogenesis [5] and as delivery methods, polymeric vectors can achieve protection, effective intracellular delivery, tumor targeting and controlled release of CRISPR/Cas9 system.

Several researches convinced that polymeric vector based CRISPR/Cas9 system targeting proto-oncogenes could disrupt the target gene and suppress tumor growth. Leong et al. developed a self-assembled micelle to delivery CRISPR/Cas9 system targeting human papillomavirus (HPV) E7 oncogene [38]. HPV E7 can inhibit a kind of cell-cycle negative regulation protein-retinoblastoma protein (Rb) and lead to uncontrollable cell proliferation [70]. The plasmid which encoded Cas9 and sgRNA targeting HPV E7 was condensed in a polymeric micelle composed of Pluronic F127 and quaternary ammonium-modified poly (propylene oxide). The micelles showed effective HPV E7 disruption

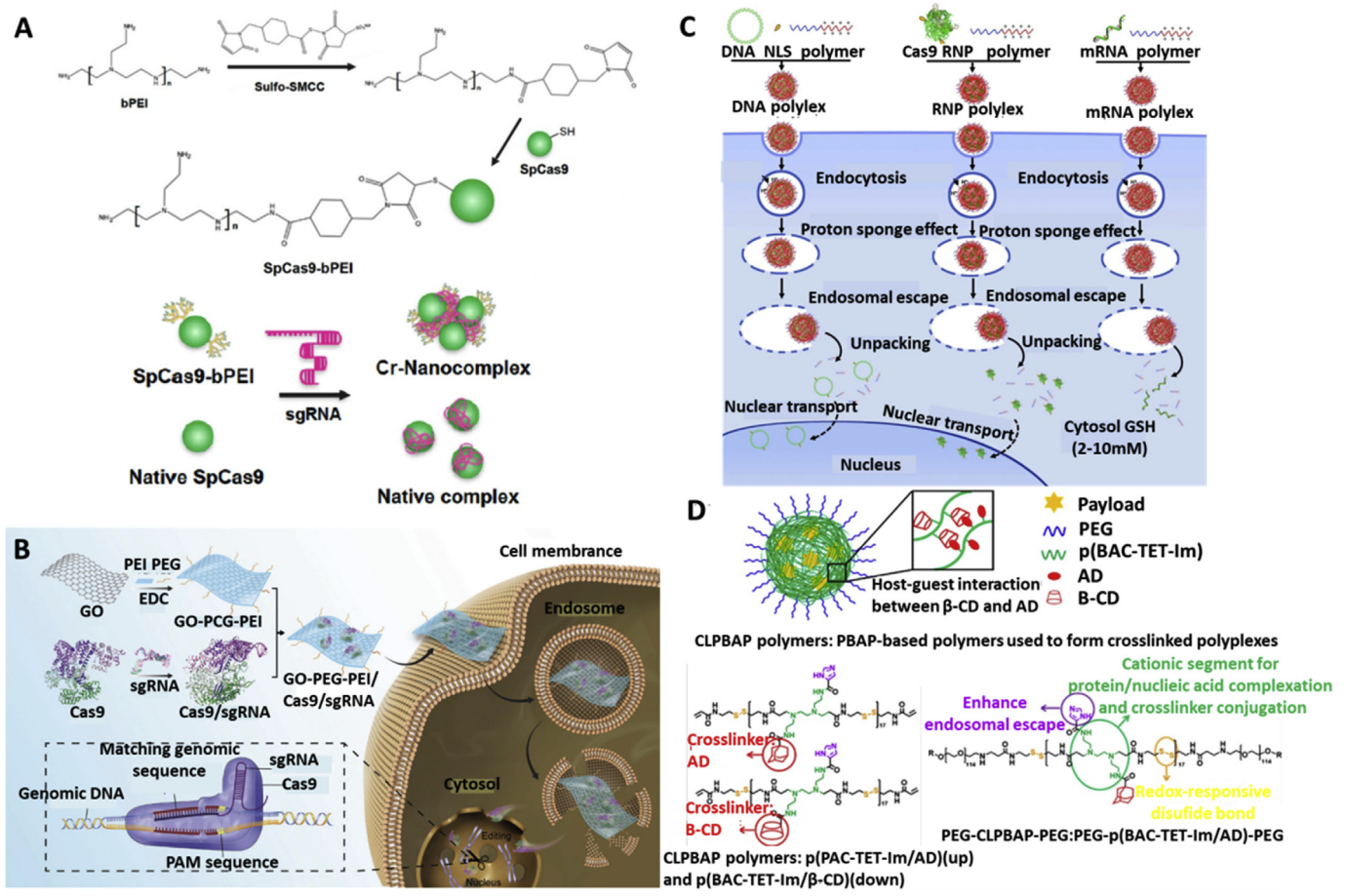


Fig. 7. (A) SpCas9 was modified with branched PEI, the spCas9-bPEI condensed sgRNA to form CRISPR nanocomplex. (Copyright 2018, American Chemical Society) [46] (B) Illustration of the intracellular delivery of GO-PEG-PEI based Cas9/sgRNA delivery system. Cas9/sgRNA complex was loaded in GO-PEG-PEI through electrostatic and π-stacking interactions. After taken up through endocytosis, the complex escaped from endosome and transported into nucleus where Cas9/sgRNA exhibited their gene editing efficiency. (Copyright 2018, Royal Society of Chemistry) [47] (C) Schematic of the intracellular delivery of the DNA, mRNA, and Cas9 RNP. After the polyplexes were taken up, the polymers were able to achieve effective endosomal escape owing to positive charged imidazole groups. When they appeared in the cytosol, the disulfide bonds were cleaved by GSH and the cationic polymers were converted into neutral polymers, thus releasing the payloads. (Copyright 2018, American Chemical Society) [49] (D) Illustration of polymeric complexes and chemical structures of the redox-responsive polymers, the cationic segments were for the conjugation of payloads, imidazole groups enabled rapid endosomal escape, disulfide bonds endowed it with redox-responsiveness and crosslinkers enhanced the stability of the complexes. (Copyright 2018, American Chemical Society) [48].

and SKOV3 tumor growth suppression, as showed in Fig. 8A. In a research carried out by Wang et al. [41], researchers chose BCR-ABL gene as target site, cationic lipid-assisted PLGA nanoparticles as delivery vectors to perform gene knockdown in chronic myeloid leukemia (CML) cells. It is known that BCR-ABL fusion protein is a kind of consititutive tyrosine kinase that plays a key role in the development of CML. It activates a series of proteins and accelerates the proliferation of myeloid cells, resulting in CML. Thus the disruption of BCR-ABL gene is an effective way to treat CML, as showed in Fig. 8B [71]. In another research performed by Leong, oncogene polo-like kinase 1 (Plk1) [72] was targeted by a kind of cell-penetrating polypeptide based CRISPR/Cas9 system. The polypeptide was modified with cationic side groups to condense Cas9 plasmid and sgRNA, forming a kind of polypeptide nanoparticles. The nanoparticle could be taken up by cells through endocytosis as well as pore formation, leading to enhanced cellular uptake.

Moreover, cationic polymeric vectors can also achieve tumor cell targeting ability and responsive release of its payloads. Zhang et al. designed a kind of lipopolymer targeting angiogenic factor -vascular endothelial growth factor A (VEGFA) to treat Osteosarcoma. Under the stimulation of VEGFA, endothelial cells are activated to promote agiogenesis in tumor tissue to delivery nutrient and oxygen [73]. In this research, the CRISPR/Cas9 plasmid was condensed in PEG-PEI-Cholesterol (PPC) lipopolymer that had been proven an effective and safe vector in clinical test, and the lipopolymer was further modified with OS cell-specific aptamer-LC09 [74] to achieve tumor cell-specific delivery. In another research carried out by Wei et al., they targeted an oncogene the mutT homolog1 (MTH1) [75] with a “core-shell” artificial virus [37]. The artificial virus was conformed of fluorinated LMWPEI covered by with multifunctional RGD-R8-PEG-HA (RRPH) copolymer, as showed in Fig. 8C. The RGD-R8 peptide endowed the vector with targeting ability to tumor and tumor vessel which highly express a kind of integrin receptor as shown in Fig. 8D [76], and the HA segment endowed it with charge reverse in tumor tissue that overexpressed hyaluronidase (HAase) to enhance cellular uptake.

3.2. Cationic polymer based CRISPR/Cas9 system in gene therapy of other disease

Except for cancer gene therapy, polymer based CRISPR/Cas9 delivery system also shows great potential in treating many other diseases. In a series of researches performed by Wang et al., they utilized cationic-lipid assisted PLGA nanoparticles (CLANs) for delivery of CRISPR/Cas9 system in macrophages and B cells to treat type 2 diabetes, rheumatoid arthritis or ameliorate inflammatory diseases, as shown in Fig. 9A. Those CLANs successfully achieved disruption of

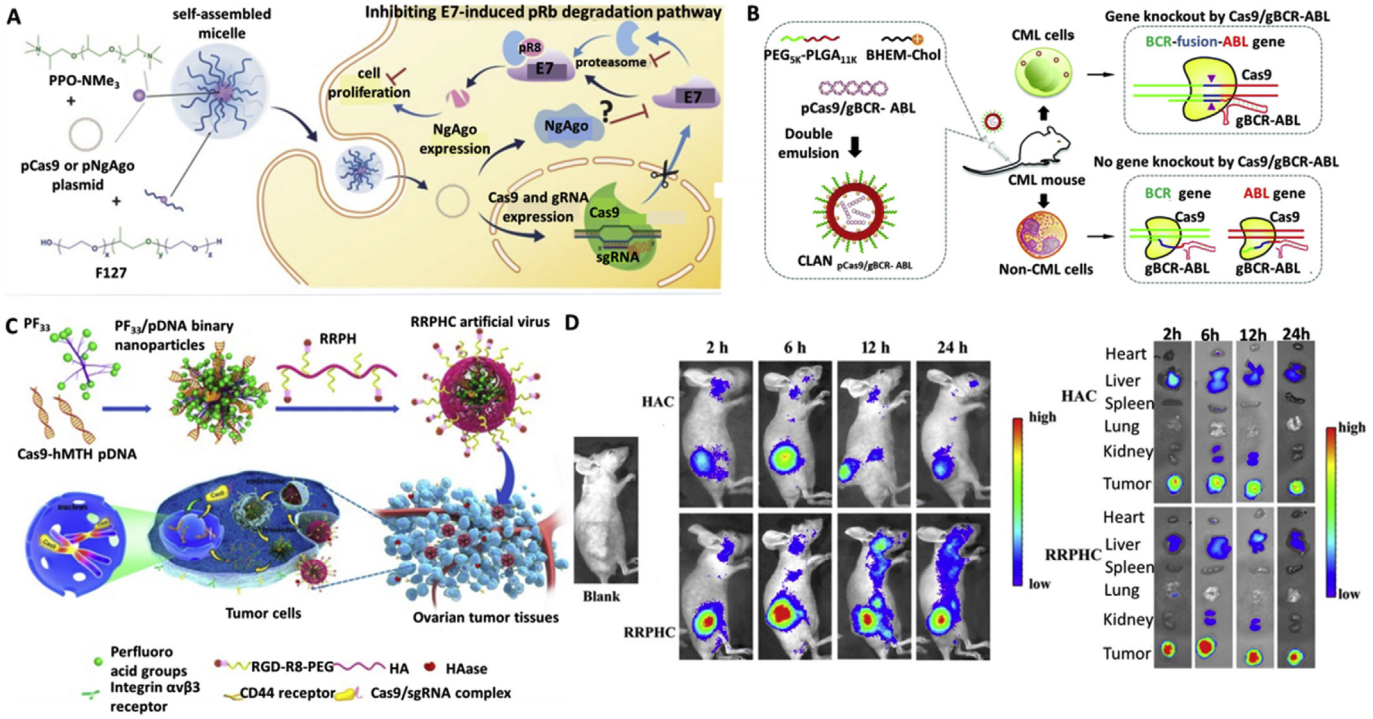


Fig. 8. (A) Diagram of HPV oncogene manipulated with the micelle. The micelle was composed of Pluronic F127 and a kind of quaternary ammonium-modified poly (propylene oxide), condensing Cas9 plasmid and sgRNA. The plasmid CRISPR system could knock down E7 gene, thus inhabited E7 induced Rb degration. (Copyright 2018, Wiley-VCH) [38]; (B) Schematic diagram of CLANs targeting BCR-ABL gene to treat CML. PCas9/gBCR-ABL was encapsulated into cationic-lipid assisted PLGA nanoparticles. The nanoparticles specifically recognized the BCR-ABL fusion gene in CML cells but not BCR or ABL gene in normal cells, thus exhibit therapeutic effect on CML and limited side effects. (Copyright 2018, the Royal Society of Chemistry) [41]; (C)The diagram of the composition of artificial virus and its targeting ability to tumor cells. Plasmid Cas9/sgRNA MTH1 was condensed by fluorinated LMWPEI then covered by RRPH. RGGD-R8 peptide and HA respectively targeted to integrin αvβ3 receptors and CD44 receptors. (Copyright 2016, American Chemical Society) [37]; (D)Analysis of tumor targeting ability of artificial virus *in vivo*. The left figure is a series of *in vivo* fluorescence imaging of the SKOV3 tumor-bearing nude mice after respectively intravenous injected of RRPH coated and HA coated PF33/Cas9-hMTH1 nanoparticles. The right figure is *ex vivo* fluorescence imaging of the tumors and other tissues gained from the tumor-bearing nude mice. Both figures showed that RRPH coated nanoparticles exhibited high tumor-targeting ability. (Copyright 2016, American Chemical Society) [37].

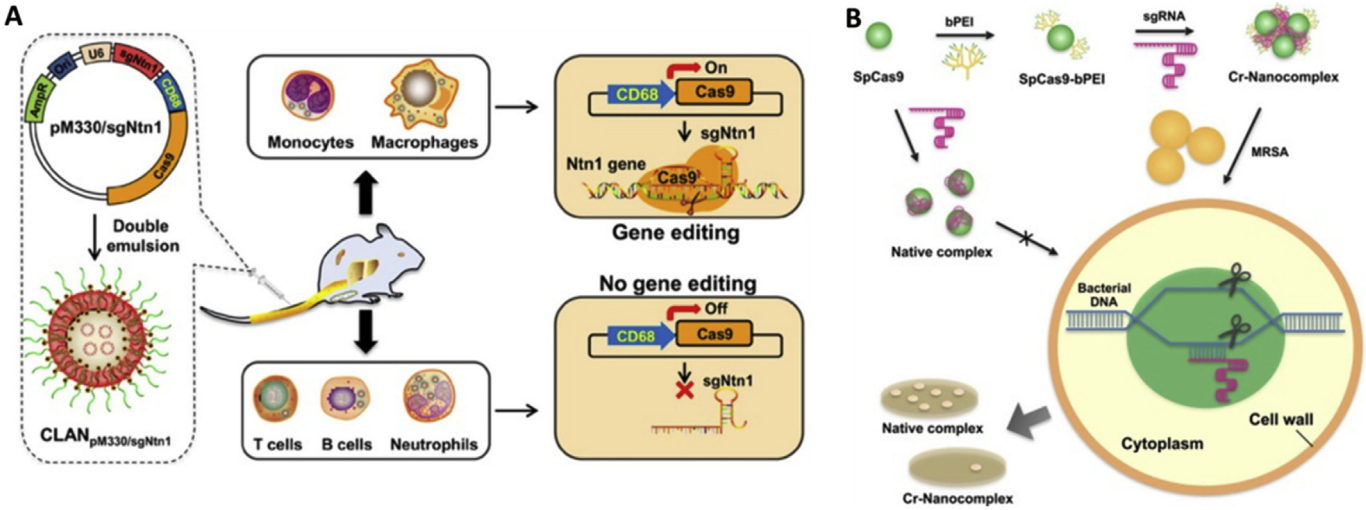


Fig. 9. (A) The schematic diagram of cationic-lipid assisted PLGA nanoparticles induced macrophages targeted gene disruption. The CRISPR/Cas9 plasmid contained cell-specific promotor-human CD68 promoter, thus specifically expressed in macrophages and led to gene knockdown. (Copyright 2018, American Chemical Society) [45]; (B) Schematic depiction shows CRISPR-nanoparticles disrupting bacterial antibiotic resistance gene. SpCas9 was modified with branched PEI to prepare the spCas9-bPEI and native spCas9 was chosen as comparison, both of them condensed sgRNA targeting mecA in bacterial, resulting in CRISPR nanocomplex and native complex. The result showed that the PEI modified Cas9 induced more remarkable inhabitation of bacterial growth compared with native complex. (Copyright 2018, American Chemical Society) [46].

Ntn1 gene in macrophages with CRISPR/Cas9 plasmid for type 2 diabetes treatment. To avoid undesired gene disrupting in other cells, the Cas9 plasmid was designed with CD68 promoter which was able to drive specific plasmid expression in macrophages, thus achieve cell-specific gene editing [43]. In another research, a library of CLANs were fabricated to optimize delivery of CRISPR/Cas9 plasmids to B cells and finally CLANs with 40.30% PEG density and 12.2 mV surface charge were chosen. The CLAN based CRISPR/Cas9 plasmid system knocked down B-cell activating factor receptor (BAFFR) [77] gene and B220 gene in B cells and showed alleviation of rheumatoid arthritis in mice [42]. The nanoparticles were also qualified to delivery Cas9 mRNA/sgRNA. In the research, CLANs were optimized to disrupt NLRP3 gene in macrophages. The knockout of NLRP3 showed potential in ameliorating type2 diabetes, peritonitis as well as septic shock [45]. In addition to mammalian cells, polymer based CRISPR/Cas9 delivery system was also used in bacterial. Chung et al. utilized bPEI-derivatized Cas9 to complex with sgRNA targeting methicillin resistance gene-mecA. This strategy could achieve effective delivery into bacterial with minimal amount of vectors, which was hard due to the cell wall. The delivery system was introduced into methicillin-resistant *Staphylococcus Aureus* (MRSA) and resulted in distinct growth inhabitation, as shown in Fig. 9B [46].

4. Conclusion and perspectives

The development of this novel CRISPR/Cas9 gene editing platform has revolutionized the field of gene therapy. Researchers have revealed its application in monogenic disorders (*i.e.*, cataracts, hereditary tyrosinemia) as well as in non-monogenetic diseases (*i.e.*, hypercholesterolaemia, cancers). To achieve its function, the effective intracellular delivery of CRISPR/Cas9 components is necessary and great effort has been made in developing novel delivery strategies. Among them, cationic polymeric vectors stand out from the crowd for their unique properties (*i.e.*, low immunogenicity and carcinogenicity, less limitation in payloads size, or protection of cargos). Besides to those basic properties, the most exciting part is the further modification of the polymers (*i.e.*, for targeting ability). Connected with specific peptides, antibodies, aptamers or other molecules, polymers are able to deliver their cargos to specific cell types [78]. Targeted delivery can selectively accumulate the cargos in the disease area, thus reduces the related side effects. Considering the potential risk of off-targeting effect of CRISPR/Cas9 system, accurate targeted delivery is necessary for its clinical application. Current researches have achieved targeted delivery of CRISPR/Cas9 system to tumor cells with polymeric vectors in form of aptamer-functionalized lipopolymer [36] or “core-shell” artificial virus [37], while further researches can expand the application in other cells and tissues like immune cells, cardiovascular epithelial cells, liver and so on. Stimulus-sensitive release system is another effective tool in polymeric delivery vectors. Through modification, polymers can response to diverse stimulus like PH, redox, temperature, light, enzyme and so forth [79]. Those responses endow polymers with ability to retain their cargos in circulation, concentrate at the desired site and desired time, enhance cellular internalization and achieve effective drug release [80]. According to current researches in polymeric vectors for CRISPR/Cas9 delivery, the “core-shell” artificial virus can response to the hyaluronidase overexpressed in tumor tissue to achieve charge reverse and enhanced cellular uptake of CRISPR/Cas9 plasmid [37]. And the redox-responsive copolymers can response to GSH in cytoplasm to effectively release CRISPR/Cas9 ribonucleoproteins [48,49]. The application of stimulus-sensitive polymeric delivery system is worth trying in further CRISPR/Cas9 based gene therapy. In addition, polymeric architectures like hydrogels and micro or nanospheres are able to provide additional advantage of sustained release [81,82]. These structures serve as reservoirs of therapeutic substances to keep long-term effective plasma concentration, resulting in sustained therapeutic effect and avoiding the need of repeated administration. Moreover, stimulus-sensitive release and targeting ability can also be achieved in those architectures with further modification of the polymeric units, endowing them with accurate, efficient and long-term therapeutic effect. The application of those polymeric structures in CRISPR/Cas9 delivery is an attracting field worthy of further research.

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